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Comparison of cell-specific activity between free-living and attached bacteria using isolates and natural assemblages

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Abstract

Marine snow aggregates are microbial hotspots that support high bacterial abundance and activities. We conducted laboratory experiments to compare cell-specific bacterial protein production (BPP) and protease activity between free-living and attached bacteria. Natural bacterial assemblages attached to model aggregates (agar spheres) had threefold higher BPP and two orders of magnitude higher protease activity than their free-living counterpart. These observations could be explained by preferential colonization of the agar spheres by bacteria with inherently higher metabolic activity and/or individual bacteria increasing their metabolism upon attachment to surfaces. In subsequent experiments, we used four strains of marine snow bacteria isolates to test the hypothesis that bacteria could up- and down-regulate their metabolism while on and off an aggregate. The protease activity of attached bacteria was 10–20 times higher than that of free-living bacteria, indicating that the individual strains could increase their protease activity within a short time (2 h) upon attachment to surfaces. Agar spheres with embedded diatom cells were colonized faster than plain agar spheres and the attached bacteria were clustered around the agar-embedded diatom cells, indicating a chemosensing response. Increased protease activity and BPP allow attached bacteria to quickly exploit aggregate resources upon attachment, which may accelerate remineralization of marine snow and reduce the downward carbon fluxes.

Introduction

Marine snow aggregates are 'microbial hotspots' characterized by high microbial abundance and activity relative to the surrounding water (Azam, 1998; Simon *et al.*, 2002). Commonly observed elevated bacterial growth rate and enzymatic activity associated with aggregates suggests that they support high bacterial production, which in turn may accelerate the dissolution and disintegration of the aggregates (Smith *et al.*, 1992; but also see Alldredge *et al.*, 1986). On a per unit volume basis, bacteria are several orders of magnitude more abundant on marine snow aggregates than in the surrounding water (Simon *et al.*, 2002). Several studies have also shown that the cell-specific enzymatic activity of attached bacteria is at times one to two orders of magnitude higher than free-living bacteria, particularly at the peak of an algal bloom (Karner & Herndl, 1992; Smith *et al.*, 1995; Riemann *et al.*, 2000; Grossart *et al.*, 2003; but also see Martinez *et al.*, 1996). It is, however, not known whether the high activity of attached bacteria is a result of the aggregates being colonized by bacteria with inherently high metabolic activity, or bacteria increasing their metabolism after encounter with the aggregates. For bacteria, a successful encounter with an aggregate in the ocean can be a rare event, and the estimated average 'searching time' for an aggregate in the upper ocean ranges from 0.02 to 12 days (Kiørboe *et al.*, 2002). For bacteria that specialize in living on marine snow aggregates, it would be beneficial for them to turn down their metabolism when in the suspension phase, and turn up their metabolism only when an aggregate is detected or after a successful encounter with an aggregate. We conducted a series of laboratory experiments, using natural bacterial assemblages as well as marine snow bacteria isolates, to compare the colonization behavior of the bacteria in the presence or absence of artificial 'diatom aggregates'. We also compared the enzymatic activity and production rate of bacteria on and off an aggregate to test whether bacteria show different activities in the free-living vs. the attached stage.

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Materials and methods

Experiments with natural bacterial assemblage

Seawater was collected from the surface of Gullmarsfjordens and immediately returned to the Kristineberg Marine Research Station (Sweden). The seawater was passed through a 10-μm sieve to remove large particulates, and the natural bacterial community was concentrated using a 0.16-μm tangential flow system (Filtron). Concentrated bacterial assemblages were transferred to 500-mL beakers for experiments. Aggregates were simulated with 4-mm diameter agar spheres prepared according to Kiørboe *et al.* (2002) with and without marine broth (MB) enrichment. Agar spheres were suspended on glass needles and immersed in the concentrated bacterial assemblages for 2 h. Afterward, agar spheres with attached bacteria and aliquots of the surrounding water (in triplicate) were removed for bacteria quantification by DAPI direct count (Porter & Feig, 1980); additional agar spheres and water aliquots were taken for measuring free-living and attached bacterial protein production (BPP) and protease activity as described below.

To measure BPP and protease activity of attached bacteria, aliquots from the bacterial suspensions were filtered through 0.2-μm membrane filters and 3 mL of each filtrate were added to a cuvette. For each treatment, three precolonized agar spheres were suspended on glass needles in cuvettes with filtrate (in triplicate). Aliquots of the bacterial suspensions were added directly to a separate set of cuvettes (3 mL in triplicate) to measure BPP and protease activity of free-living bacteria. All cuvettes were incubated with substrates for either BPP or protease measurements at 20 °C in the dark for 1 h before measurements (see below).

Rates of BPP were determined by bacterial incorporation of ^{14}[C]-leucine (^{14}C-Leu, Simon & Azam, 1989). Triplicates and a formalin-sacrificed control were incubated with ^{14}C-Leu (312 mCi mmol^{-1}, Amersham, England) at a final concentration of 50 nmol L^{-1} to ensure saturation of uptake systems of both free-living and attached bacteria. Standard deviation of triplicate measurements was usually $< 15\%$. Biomass production was calculated according to Simon & Azam (1989). To convert protein production to carbon production, a conversion factor of 0.86 was used.

Protease activity was measured using L-leucine-methyl coumarinyl amide (Leu-MCA) as a substrate analogue (Hoppe, 1983). Sterile agar spheres and aliquots of sterile filtered seawater were used as control for attached and free-living bacteria, respectively. The final concentration of the added fluorogenic substrate was 0.1 mM, which ensured maximum hydrolysis as determined by saturation kinetics. The fluorescence in the cuvette was read on a fluorometer (Kontron, excitation 365 nm, emission 455 nm). A calibration curve for the enzyme assay was prepared by measuring the fluorescence over a range of substrate concentrations.

Experiments with marine snow bacteria isolates

The marine snow bacteria HP4, HP11, HP15b, and HP33 were originally isolated from marine snow aggregates (Grossart *et al.*, 2004) and maintained on 50% MB agar. Before the experiments, inocula of the isolates were resuspended in MB solution in test tubes and allowed to regain motility and grow to high density overnight. Actively growing bacteria were diluted with sterile aged seawater (1-year old North Sea water) to a concentration of *c.* 10^6 cells mL^{-1} in 500-mL beakers for experiments as described below. The colonization rate of the bacteria onto model particles was measured using 4-mm diameter agar spheres following Kiørboe *et al.* (2002). We prepared two types of agar spheres: (1) plain agar spheres were made of 2.5% (w/v) ultra-pure agar to minimize introduction of organic contaminants during the experiments. (2) To simulate diatom-derived marine snow aggregates, we prepared a second batch of agar spheres by mixing an axenic culture of the diatom *Thalassiosira weissflogii* (CCMP1053; *c.* 10^6 cells mL^{-1}) with 2.5% ultra-pure warm agar. The warm agar was then molded and solidified into 4-mm diameter agar spheres with the axenic diatom cells embedded inside. Using diatom-agar spheres enabled us to circumvent methodological problems associated with using real diatom aggregates such as physical disintegration of the aggregates during handling and convoluted aggregate surfaces that make bacterial quantification difficult. The embedded diatom cells could release chemical signals through the agar matrix, whereas the agar matrix provided a stable structure to hold the diatom cells together and a uniform surface for quantifying attached bacteria. Both plain and diatom-agar spheres were suspended on glass needles and immersed in bacterial suspensions for 2 h. At certain time intervals, triplicate agar spheres were removed and attached bacteria were quantified by DAPI direct count. Aliquots of ambient water were taken at the beginning and the end of the experiments for quantifying free-living bacteria. Attached bacterial abundance per sphere was normalized to ambient bacterial concentration. The mathematical model of Kiørboe *et al.* (2002) was fitted to the observations. Protease activities of attached and free-living bacteria were measured in the same manner as described before.

Results

Natural bacterial assemblage

Cell-specific protein production (BPP) of free-living bacteria was much higher when agar spheres (with or without MB

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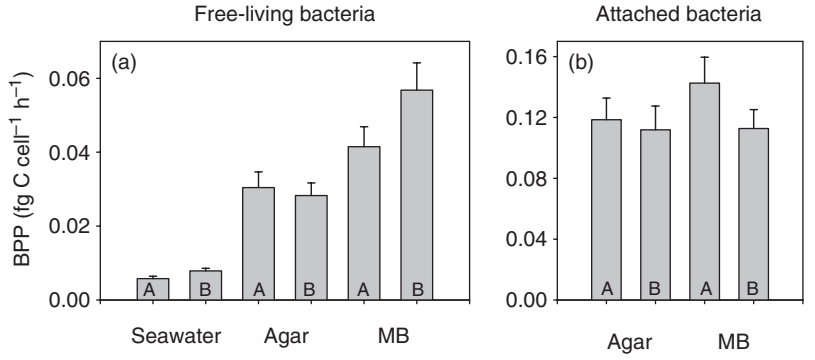

Fig. 1. Agar sphere experiments: Cell-specific protein production of (a) free-living and (b) attached bacteria after 68 h of incubation in either plain seawater (Seawater), seawater with agar spheres (Agar), or seawater with MB-enriched agar spheres (MB). All measurements were performed in two parallel experiments (A+B).

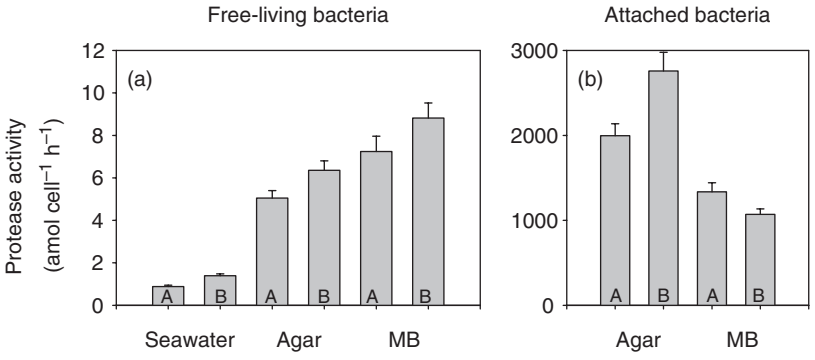

Fig. 2. Agar sphere experiments: Cell-specific protease activities of (a) free-living and (b) attached bacteria after 68 h of incubation in either plain seawater (Seawater), seawater with agar spheres (Agar), or seawater with MB-enriched agar spheres (MB). All measurements were performed in two parallel experiments (A+B).

enrichment) were present (Fig. 1a). Bacteria attached to agar spheres (Fig. 1b) had an almost threefold higher cell-specific BPP than the free-living cells. There was no difference between agar spheres with and without MB enrichment, indicating that the cell-specific BPP of the attached bacteria was almost at maximum in both treatments.

The cell-specific protease activity of the natural bacterial assemblage was significantly increased in the presence of agar spheres (Fig. 2a). The cell-specific protease activities of attached bacteria were up to two orders of magnitude higher than free-living bacteria (Fig. 2b). Between the two types of agar spheres, enrichment with MB resulted in lower protease activities, which may indicate the presence of higher monomer and oligomer concentrations on the MB-enriched agar spheres.

Marine snow bacteria isolates

The colonization process followed the pattern described by Kiørboe *et al.* (2002). Attached bacterial abundance on both plain and diatom-agar spheres increased with time. Bacteria were randomly distributed on the surfaces of plain agar spheres. For diatom-agar spheres, however, the bacteria clustered around the embedded diatoms, indicating a chemosensing response. All four strains colonized diatom-agar spheres faster, and the effect was more pronounced for HP4 and HP15b (Fig. 3).

The cell-specific protease activity of free-living bacteria was relatively low for HP4, HP11, and HP15b, ranging between 420 and 980 amol cell^{-1} h^{-1}; in contrast, the protease activity of free-living HP33 was much higher, at 8400 amol cell^{-1} h^{-1} (Fig. 4). Protease activities of attached bacteria for HP4, HP11, and HP15b were all significantly higher, *c.* 10–20 times that of the free-living counterpart (*t*-test, $P < 0.05$). However, there was no significant difference between plain spheres and diatom-agar spheres for HP4 and HP11. Surprisingly, plain spheres yielded a significantly higher protease activity than diatom-agar spheres for HP15b (*t*-test, $P < 0.05$). There was no significant difference in protease activity between attached and free-living bacteria for HP33. The average protease activity of attached HP33 was one to two orders of magnitude higher than the other strains, but also the measured activity was highly variable (Fig. 4).

Discussion

Aggregates in aquatic environments are often characterized by high bacterial abundance, growth rate, and enzymatic activity relative to the surrounding water (Simon *et al.*, 2002). Observations on natural aggregates show that the attached bacterial community composition tends to differ from that in the surrounding water (Riemann *et al.*, 2000; Kirchman, 2002; Grossart *et al.*, 2005, 2006). As such,

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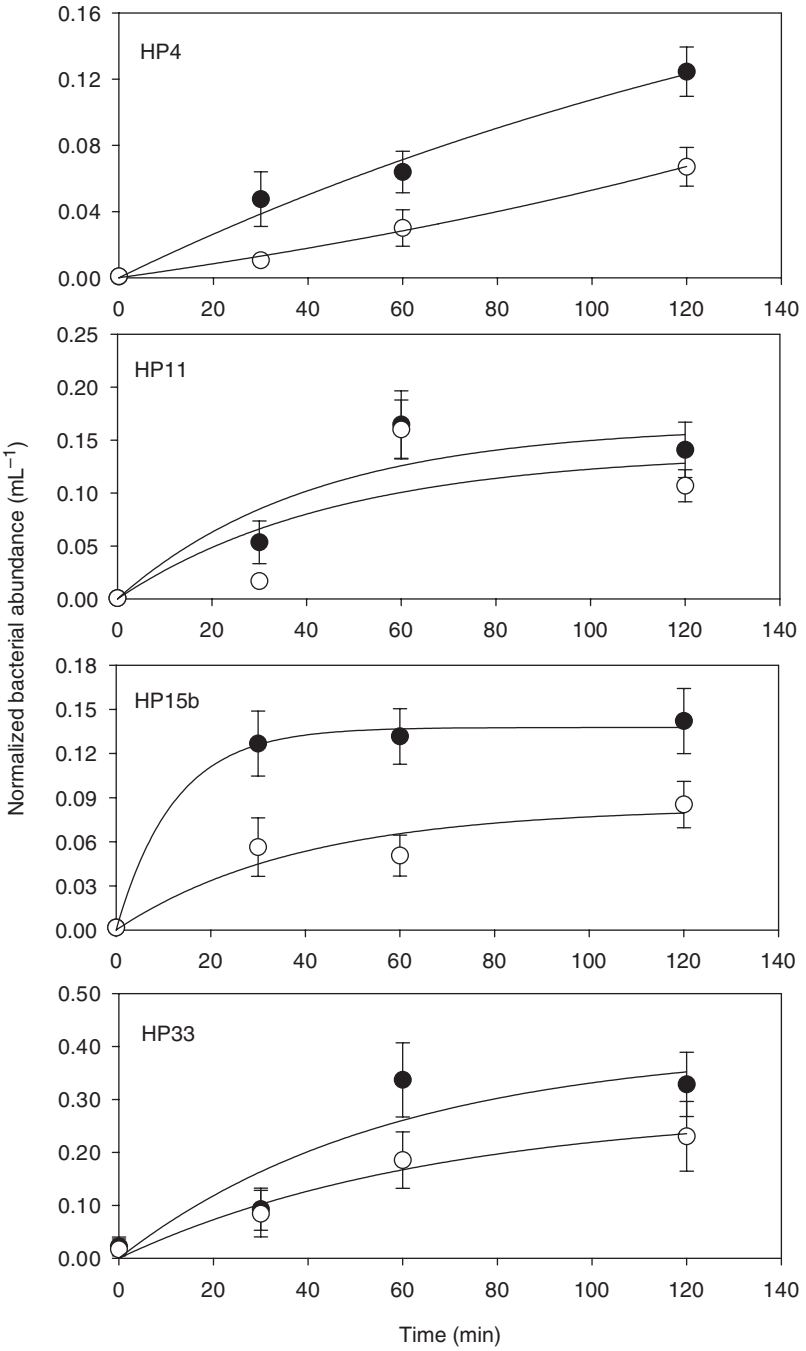

Fig. 3. Colonization of 4-mm diameter plain (○) or diatom-agar spheres (●) in still water by four strains of marine snow bacteria. Bacterial abundances on the spheres were normalized to ambient bacterial concentrations. Error bars represent SDs of 10 measurements. Solid lines represent model fits according to Kiørboe *et al*. (2002).

elevated growth rate and enzymatic activity could be due to preferential colonization of the aggregates by bacteria with an inherently high metabolic activity and/or that individual bacteria increase their activity upon attachment to aggregates. In a previous study (Grossart *et al*., 2006), we showed that the community composition of free-living and attached bacteria increasingly differed throughout the formation of diatom aggregates. The present study revealed that the presence of agar spheres stimulated enzymatic activities and production of both free-living and attached bacteria. These observations suggest that microbial processes on marine snow particles do not exclusively affect the attached bacterial community. Uncoupling between POM hydrolysis and subsequent uptake of hydrolysis products by attached bacteria lead to the release of DOM from marine aggregates into the surrounding water (Smith *et al*., 1992; Grossart &

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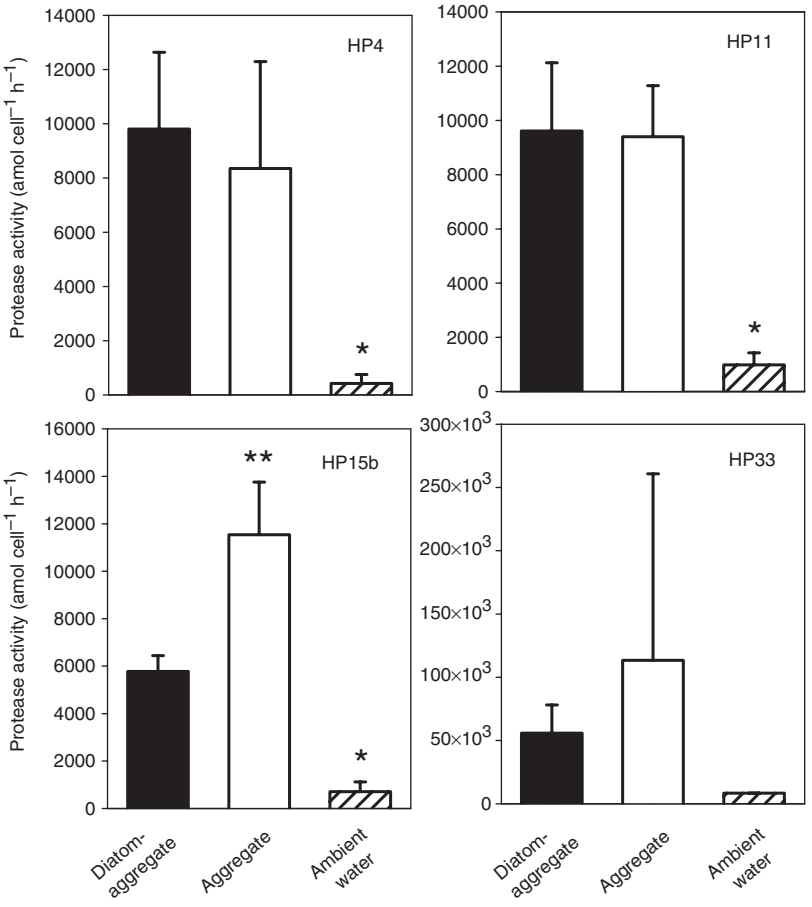

Fig. 4. Cell-specific protease activity of four strains of marine snow bacteria on plain spheres, diatom-agar spheres, and in ambient water (mean+SD; $n = 3$). Asterisks indicate significant differences (one way-ANOVA followed by Tukey test, $P < 0.05$).

Simon, 1998; Grossart & Ploug, 2001), which also stimulate hydrolytic activities and growth of free-living bacteria (Grossart & Simon, 1998; Kiørboe *et al.*, 2001).

Natural bacterial assemblages attached to agar spheres had an almost threefold higher cell-specific BPP and two orders of magnitude higher protease activity than free-living bacteria (Figs 1 and 2). At first, it seems surprising that attached bacteria on MB-enriched agar spheres expressed reduced protease activities but similar BPP rates. The lower proteolytic activity, however, may point to a higher availability of mono- and oligomers originating from MB, which do not require prior enzymatic hydrolysis (Grossart & Ploug, 2001). The similar and very high cell-specific BPP on both plain and MB-enriched agar spheres demonstrates that bacteria on both types of spheres had reached their maximum growth rates.

Even though attached bacteria expressed much higher cell-specific BPP and protease activity, in complex bacterial communities it remains questionable whether bacteria change their physiological behavior during attachment to aggregates. Therefore, in subsequent laboratory experiments, we used defined marine snow bacteria isolates to further test the hypothesis that individual bacteria may turn on their metabolic activity upon attachment to aggregates. Colonization of plain agar spheres by the bacteria suggests that the bacteria had a tendency to colonize particle surfaces even in the absence of phytoplankton cells, although the colonization rate tended to be higher with diatom-agar spheres. These results suggest that the bacteria reacted chemotactically to the embedded diatom cells, which may have also leaked dissolved organic matter. Consistent with this hypothesis, microscopic observations showed clustering of bacteria around embedded diatom cells. Interestingly, despite the higher colonization rate with diatom-agar spheres, the protease activity of attached bacteria was not enhanced relative to plain spheres. Contrary to our expectation, attached HP15b on plain spheres had higher protease activity than those on diatom-agar spheres. Because the bacteria tended to form clusters on diatom-agar spheres, the individual cells might have less exposure to ambient substrate relative to plain spheres where the bacteria were more evenly distributed, resulting in lower measured protease activity. Nevertheless, the attached bacteria had significantly higher protease activity than the free-living bacteria, with the exception of HP33 where the measured protease activity was highly variable. The high variability in the measured

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protease activity of HP33 may be linked to the fact that these bacteria are not necessarily permanently attached to the spheres. Many bacteria can jump on and off the aggregates many times during the incubation (Kiørboe *et al.*, 2002), which may also lead to increased protease activity of free-living bacteria being temporarily detached from the spheres. Because the plain spheres in this set of experiments were made of ultra-pure agar that did not contain any protein, the increased protease activity was not a response to protein sources but triggered by mere attachment to surfaces. This is consistent with the observations of Taylor & Gulnick (1996), who showed that the presence of inert, organic-free surfaces (glass beads) enhanced proteolyic enzyme activities and leucine incorporation by salt marsh bacteria even though few bacteria were permanently attached to the surfaces.

The protease activity of attached bacteria isolates in our experiments was 5.8–113 $\times 10^3$ amol cell^{-1} h^{-1}, substantially higher than some reported values for bacterial assemblages on phytoplankton aggregates (Martinez *et al.*, 1996; Riemann *et al.*, 2000), but comparable to the maximum attached bacteria protease activity during a diatom bloom in a mesocosm (Smith *et al.*, 1995). On the other hand, our free-living bacteria protease activity was an order of magnitude higher than that of free-living natural bacterial assemblages used by us and other investigators (e.g. Smith *et al.*, 1995; Martinez *et al.*, 1996; Riemann *et al.*, 2000), indicating either inherently high strain-specific protease activity for our isolates, or the fact that our bacteria were maintained in active growth before experiments and interact with the spheres.

In summary, our results show that individual bacteria isolates could up- or down-regulate their protease activity, hence BPP, while on or off an aggregate. This adjustment apparently can occur very rapidly – within 2 h (duration of precolonization of the agar spheres). Because encountering an aggregate could be a rare event in the open ocean, the ability to rapidly attach and adjust enzymatic activity allows the bacteria to conserve energy between successful encounters, while maximizing exploitation of aggregate resources upon attachment. Up-regulation of enzyme activity upon colonization of an aggregate may be mediated by quorum-sensing and signal molecules, which have been demonstrated in marine snow bacteria (Gram *et al.*, 2002). Efficient aggregate dissolution and biomass formation by bacteria counteract the export of carbon and nutrients to the sediments and, thus, have profound consequences for fluxes of organic matter and nutrients in the sea.

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